Structure and apoprotein linkages of phycourobilin

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R-Phycoerythrin contains two covalently bound bilin prosthetic groups, phycoerythrobilin and phycourobilin. The two chromophore types were separated as their peptide-bound derivatives by subjecting tryptic digests of R-phycoerythrin to adsorption chromatography on Sephadex G-25. The structure and apoprotein linkages of the bound phycoerythrobilin were found to be identical with those previously reported for this phycobilin [Killilea, O'Carra & Murphy (1980) *Biochem. J.* **187**, 311–320]. Phycourobilin is a tetrapyrrole, containing no oxo bridges and has the same order of side chains as IXα bilins. The chromophore is linked to the peptide through two and possibly three of its pyrrole rings. One linkage possibly consists of an ester bond between the hydroxy group of a serine residue and the propionic acid side chain of one of the inner rings. The second linkage is a labile thioether bond between a cysteine residue and the C_2 side chain of pyrrole ring A. The third linkage is a stable thioether bond between a cysteine residue and the α-carbon atom of the C_2 side chain of pyrrole ring D. Ring D is unsaturated and is attached to ring C through a saturated carbon bridge. Rings B and C have a conjugated system of five bonds, as found in other urobilinoid pigments. Ring A is attached to ring B via a saturated carbon bridge. Both of the α-positions of ring A are in the reduced state, but the ring does contain an unsaturated centre (probably a double bond between the β-carbon and the ring nitrogen atom). The presence of this double bond and its isomerization into the bridge position between rings A and B would explain the extension of the conjugated system of phycourobilin to that of a phycoerythrobilinoid/rhodenoid pigment in acid or alkali.

R- and B-Phycoerythrins are complex algal biliproteins containing two different chromophores, phycoerythrobilin and phycourobilin. The existence of the phycourobilin chromophore in these biliproteins has been disputed (Chapman *et al.*, 1968), but later work re-established that phycourobilin is indeed a native chromophore of these biliproteins (O'Carra *et al.*, 1980). The structure and apoprotein linkages of phycoerythrobilin and phycocyanobilin (the chromophore found in the phycocyanins) have been reported from this laboratory (Killilea *et al.*, 1980). We now report here studies on the structure and apoprotein linkages of phycourobilin.

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Experimental

Protein preparations

R-Phycoerythrin from *Rhodymenia palmata* was isolated and purified as by O'Carra (1965). Reduction and *S*-aminoethylation of the biliprotein was carried out by following the procedure of Raftery & Cole (1966).

Cytochrome *c* was obtained from Sigma Chemical Co. The bound-haem prosthetic group was converted into the bound-verdin derivative by coupled oxidation with ascorbate by following the general procedure of Levin (1966). This derivative, called verdocytochrome *c*, was prepared as follows. To 100 mg of cytochrome *c* in 15 ml of 0.01 M-sodium phosphate buffer, pH 7.4, were added 5 ml of pyridine and 6 mg of sodium ascorbate. The resultant solution was incubated aerobically with

occasional swirling in a shallow beaker at 37°C for 2h. During this time the colour changed from brown to green. The protein was then precipitated by the addition of trichloroacetic acid (1g) and collected by centrifugation (20000g for 10min). The pellet was washed with methanol (3 × 10ml) and stored under methanol at 4°C.

Preparation and separation of the chromophore-containing tryptic-digest peptides of R-phycoerythrin

To protect the chromophores all procedures were carried out in the dark and under N_2. All buffers were de-aerated and flushed with N_2 before use. Prior modification of free thiol groups by S-aminoethylation was carried out to prevent artifact attachment of phycoerythrobilin to the polypeptide and the conversion of the bilin chromophore residues into artifact urobilinoid pigment (O'Carra et al., 1980; Killilea et al., 1980).

The tryptic digests were carried out in the presence of 2M-urea (Liu et al., 1965) to effect complete digestion of the R-phycoerythrin. Typically 200mg of S-aminoethylated R-phycoerythrin was dissolved in 5ml of 0.2M-Tris/HCl buffer, pH8.0, containing 8M-urea. This solution was then diluted with 3 vol. of 0.2M-Tris/HCl buffer, pH8.0. The protein remained in solution at the resultant molarity of urea (2M). Then 10mg of trypsin (diphenylcarbamoyl chloride-treated; Sigma Chemical Co.) in 0.4ml of 1mM-HCl was added and digestion was carried out under N_2 and toluene at 25°C for 4h. An extra 10mg of trypsin was then added, and digestion continued for a further 16h. The digest was then acidified to pH3 with 2M-HCl and centrifuged at 20000g for 20min.

Desalting and removal of the urea from the supernatant was achieved by chromatography on an Amberlite CG-50 column (2cm × 4cm) as described by Hirs (1967). The supernatant was filtered through the column at 100ml/h. The adsorbed peptides on the column were washed with 200ml of a 1% (v/v) acetic acid solution and then eluted off with 50% (v/v) acetic acid. The eluted peptides were evaporated to dryness under reduced pressure at less than 40°C and dissolved in 0.2M-acetic acid (3ml/100mg of original protein).

The phycourobilin-containing and the phyco-erythrobilin-containing peptide fractions were isolated by adsorption chromatography on Sephadex G-25 in 0.2M-acetic acid (Fig. 1). After elution from the column, the phycourobilin-containing peptide fraction was stored at −20°C under N_2 and used as soon as possible. For the determination of the concentration of the pigment an ε of 55660$M^{-1} \cdot cm^{-1}$ (S. D. Killilea, D. N. Nolan & P. O'Carra, unpublished work) at 498nm in 0.1M-HCl was used.

Acid and alkali treatments of the phycourobilin-containing peptide

Room-temperature HCl (1.0–11.6M) had no effect on the spectral properties and did not release the chromophore from the phycourobilin-containing peptide. When the chromopeptide was refluxed under N_2 in 2M-HCl the colour of the chromopeptide changed within a few minutes from a salmon-pink to a deep red. Spectral examination of the resultant solution demonstrated that the absorbance at 495nm had decreased and that at 555nm had increased. On further heating the solution reverted to the original salmon-pink colour, during which time the absorbance at 495nm returned to its original value with a concomitant decrease in that at 555nm. After 1h about 50% of the chromophore was released as a urobilinoid pigment. The first step in this sequence of reactions appears to be a conversion of phycourobilin into a pigment with phycoerythrobilinoid/rhodenoid spectral characteristics. The subsequent reversion to a urobilinoid material presumably represents the conversion of this phyco-erythrobilin derivative into an artifact urobilin under the acid conditions (O'Carra et al., 1964).

When the phycourobilin-containing peptide was subjected to methanolysis (refluxing in dry methanol saturated with dry HCl gas) under N_2 for 1h a mixture of phycoerythrobilinoid/rhodenoid and violinoid pigments, as the dimethyl esters, was released.

In methanolic 1M-KOH under N_2 at 25°C the absorption spectrum of the phycourobilin-containing peptide underwent a red-shift to give an absorption maximum at 590nm. This new absorption peak decayed slowly and irreversibly over a period of 2h with the resultant formation of a violinoid pigment. Spectral analyses of the reaction mixtures indicated that phycourobilin was first being converted into a phycoerythrobilinoid/rhodenoid pigment, which was then converted into the violin. This spectral characterization was performed on samples of the reaction mixture to which zinc acetate (final concentration 1%) was added to form the zinc complexes of the pigments. Under these conditions the visible-region absorption maxima of phycourobilin, phycoerythrobilin and violin were 510, 583 and 630nm respectively. The violin could then be released and converted into a verdinoid pigment by refluxing in methanolic 1M-KOH under N_2 for 5min.

Chromic acid degradation studies

Chromic acid degradation of free and peptide-bound bilins was carried out as by Rüdiger & O'Carra (1969). The procedure used in the case of the phycourobilin-containing peptide was as fol-

lows. To 200 μg of phycourobilin-containing pep-
tide in 0.2 ml of water was added 0.2 ml of a
solution of 1% $K_2Cr_2O_7$ in 1 M-H_2SO_4. The
mixture was incubated at 20°C for 1 h, after which
free imide material was extracted into diethyl ether
(5 × 0.2 ml). The aqueous phase was incubated at
100°C for 1 h and the imides released by this
treatment were extracted into diethyl ether
(5 × 0.2 ml). The imides were separated by t.l.c.
(Rüdiger & O'Carra, 1969; Killilea & O'Carra,
1978) and detected with chlorine/benzidine (Rein-
del & Hoppe, 1954; Ficken et al., 1956) or by the
safer starch/iodide method (Killilea & O'Carra,
1971).

*Preparation, purification and amino acid analysis of
phycourobilin-containing 'minimal chromopeptides'*

Phycourobilin-containing 'minimal chromopep-
tides' were prepared and purified from the isolated
phycourobilin-containing tryptic-digest fraction
from 200 mg of R-phycoerythrin by similar pro-
cedures to those described previously for phyco-
erythrobilin-containing 'minimal chromopeptides'
(Killilea et al., 1980). Performate oxidation,
hydrolysis and quantitative amino acid analysis
were also carried out as described previously
(Killilea et al., 1980).

Results and discussion

*Separation of the chromophore-containing tryptic-
digest peptides of R-phycoerythrin*

Resolution of the phycoerythrobilin-containing
and phycourobilin-containing tryptic-digest pep-
tides of R-phycoerythrin by electrophoresis or gel
filtration has been reported (O'Carra et al., 1980).
For the rapid large-scale separation of these
chromopeptides it was found that adsorption
chromatography on Sephadex G-25 was a conven-
ient method. A typical elution profile is shown in
Fig. 1. The phycourobilin-containing material was
eluted first as a leading salmon-pink fraction and
was well separated from the following red–violet
phycoerythrobilin-containing material. These ob-
servations are consistent with the fact that phyco-
erythrobilin has a longer conjugated system than
phycourobilin, is therefore more aromatic and
adsorbs on the Sephadex more strongly.

Integration of the phycourobilin absorbancy and
the phycoerythrobilin absorbancy over the entire
elution profile gave a phycoerythrobilin/phycouro-
bilin ratio of 1.73:1. This compares with a ratio of
1.71:1 obtained for freshly denatured undigested
R-phycoerythrin. Quantitative comparison of the
phycourobilin and phycoerythrobilin absorbance
values of the tryptic digests applied to the column
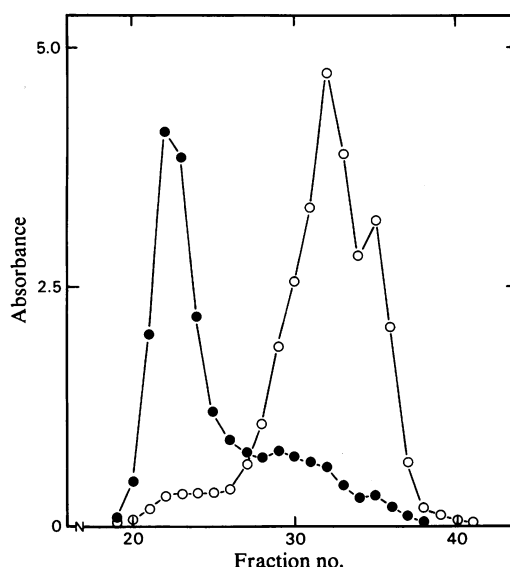with the integrated values for the eluted chromo-

Fig. 1. *Elution profile of the phycourobilin-containing (●)
and the phycoerythrobilin-containing (○) chromopeptides
from a tryptic digest of R-phycoerythrin from Sephadex
G-25*

A 3 ml portion of a concentrated tryptic digest of R-
phycoerythrin (equivalent to 100 mg of protein) was
applied to a column (2.5 cm × 52.5 cm) equilibrated
with 0.2 M-acetic acid. Chromatography was carried
out in the dark, in de-aerated, N_2-flushed 0.2 M-
acetic acid. The flow rate was 300 ml/h, and 15 ml
fractions were collected. Each fraction was made
0.1 M with respect to HCl, and its absorption
spectrum was obtained. The absorbance at 495 nm
was corrected by subtracting any absorbance in this
region due to phycoerythrobilin. Since phycouro-
bilin does not absorb at 556 nm, no reciprocal
correction was necessary.

peptide materials showed a recovery in each case
of about 85%.

The phycoerythrobilin-containing peptide has
almost the same spectral properties as denatured
C-phycoerythrin (Table 1 and Fig. 2), and the
phycourobilin-containing peptide had almost the
same spectral properties as i-urobilin (Table 1 and
Fig. 2). Each of the chromopeptides was found to
contain small amounts of material that had the
spectral properties of the other. However, these
spectral properties were not due to cross-contamin-
ation of one chromopeptide by the other. This was
determined by subjecting samples of the separated
chromopeptides to starch-gel electrophoresis, a
technique that, as mentioned above, can be used to
resolve the two chromopeptide fractions. In addi-
tion, the phycourobilin-containing peptide was
also shown to be free of the phycoerythrobilin-
containing peptide, since the imide methylvinyl-

Table 1. *Spectral absorption maxima in the visible region of denatured C- and R-phycoerythrins, the isolated tryptic-digest chromopeptides from R-phycoerythrin and i-urobilin*

The major maxima are *italicized*, the minor maxima or absorption shoulders are in parentheses. To form the zinc complex of the chromophores of C- and R-phycoerythrin, the biliproteins were dissolved in 8 M-urea containing 1% (w/v) zinc acetate, pH 7.0. The zinc complex of the bilin-containing peptides and i-urobilin were obtained in pyridine/water (1:1, v/v) containing 1% (w/v) zinc acetate.

Denatured phycoerythrin, tryptic-digest chromopeptide or bilin	Chromophore as hydrochloride in 0.1 M-HCl		Chromophore as zinc complex	
C-Phycoerythrin		*556*		*586*
R-Phycoerythrin	498	*556*	*510* (545)	*586*
Phycoerythrobilin chromopeptide	(498)	*556*	(512) (545)	*586*
Phycourobilin chromopeptide	*495*	(556)	*510*	(586)
i-Urobilin	*495*		*510*	

maleimide, which derives from ring D of phyco-erythrobilin (Rüdiger & O'Carra, 1969), was never detected in the chromic acid degradation products of the phycourobilin-containing peptide (see below). The urobilin contaminant of the phyco-erythrobilin-containing peptide was probably due to isomerization of some of the phycoerythrobilin to artifact urobilin (O'Carra et al., 1964), whereas the phycoerythrobilin contaminant of the phyco-urobilin-containing peptide was probably due to isomerization of some of the phycourobilin to a phycoerythrobilinoid/rhodenoid pigment, as discussed below.

Studies on the phycoerythrobilin-containing peptide material

As was shown above, the phycoerythrobilin-containing peptide had essentially the same spectral properties as denatured C-phycoerythrin. The chromophore of the phycoerythrobilin-containing peptide was released by treatment with conc. HCl and also by boiling methanol, or by digestion with proteinases from *Streptomyces griseus*. These results are consistent with similar studies on C- and R-phycoerythrins carried out previously (O'Carra et al., 1964; O'Carra & O'hEocha, 1966; Killilea et al., 1980). The identity of the chromophore of this tryptic-digest chromopeptide is therefore fully confirmed as native phycoerythrobilin.

Chromic acid degradative studies of the phyco-erythrobilin-containing peptide confirmed the structural integrity of the chromophore, giving the same results as were obtained by Rüdiger & O'Carra (1969) for C- and R-phycoerythrins. The structure and apoprotein linkages of this chromophore have been reported (Killilea et al., 1980).

Stability of the phycourobilin–apoprotein linkages

Efforts to release the chromophore of the phycourobilin-containing peptide by treatment with conc. HCl at room temperature, by boiling

methanol or by proteinases from *Streptomyces griseus* (Killilea et al., 1980) were unsuccessful, and more drastic reaction conditions had to be used. The chromophore of the phycourobilin-containing peptide was only released in modified form by refluxing in 2 M-HCl or methanolic 1 M-KOH or by methanolysis (see the Experimental section). No method for releasing the chromophore in intact form was discovered, and so most of the structural studies on phycourobilin were carried out on the chromophore *in situ* on the polypeptide chain.

These results are consistent with the previous observations (O'Carra et al., 1964) that the linkage of phycourobilin to the peptide chain is more stable than the phycoerythrobilin–apoprotein and phyco-cyanobilin–apoprotein linkages. O'Carra et al. (1964) postulated from indirect evidence that thioether bonding might be involved in the linkage of phycourobilin to the protein. To check out this possibility cytochrome c, in which the haem group is linked to the apoprotein through thioether bonds (Paul, 1950), and a derivative that is here named verdocytochrome c, in which biliverdin rather than haem is linked to the apoprotein through thioether bonds (see the Experimental section), were used as model systems.

As in the case of the phycourobilin-containing peptide, no pigment was released from cytochrome c or verdocytochrome c on treatment with conc. HCl at room temperature, by boiling methanol or by proteolytic digestion, but heating cytochrome c in methanolic 1 M-KOH released haematohaemin (identified by its absorption spectrum as the reduced haemochromogen in pyridine; Falk, 1964). No pigment was released on heating cytochrome c in 2 M-HCl. However, when verdo-cytochrome c was refluxed in 2 M-HCl a verdin, presumably haematobiliverdin, was released. A verdin was also released when the verdocyto-chrome c was subjected to methanolysis. These results are therefore consistent with the view that

stable thioether bonding is involved in the linkage of phycourobilin to the protein.
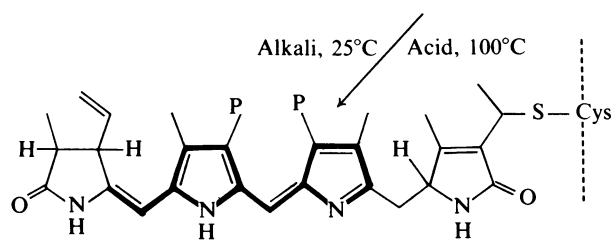
Structure of phycourobilin

Structure I (Scheme 1) is proposed for protein-bound phycourobilin. The evidence for the various structural features is as follows.

*Carbon skeleton and empirical formula.* Acid FeCl$_3$ dehydrogenation (Watson *et al.*, 1969) of the phycourobilin-containing peptide first converted the chromophore into a pigment resembling phycoerythrobilin spectrally, then into a violin and finally into a verdin. Refluxing the phycourobilin-containing peptide under N$_2$ in methanolic 1M-KOH for 5 min released the chromophore and also converted it into a green pigment with verdinoid spectral characteristics (O'Carra *et al.*, 1980). Chromic acid degradation of this verdin, as the
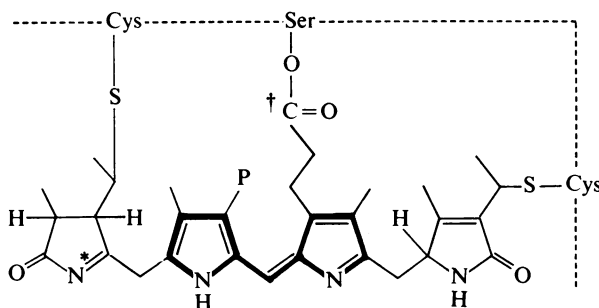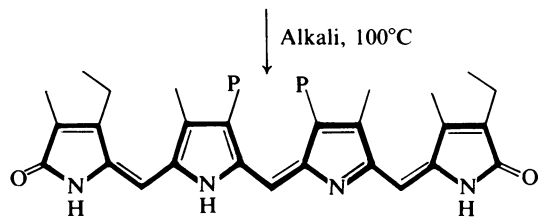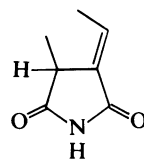

(I) Protein-bound phycourobilin

Alkali, 25°C / Acid, 100°C

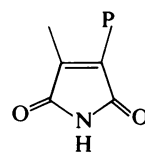(II) Phycoerythrobilinoid derivative

Alkali, 25°C
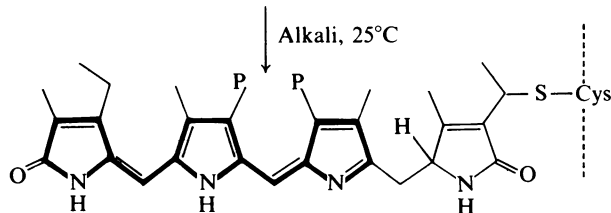
(III) Violinoid derivative

Alkali, 100°C

(IV) Mesobiliverdin IXα

(V)

(VI)

(VII)

(VIII)

P = -CH$_2$-CH$_2$-CO$_2$H
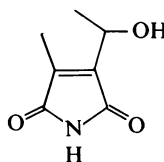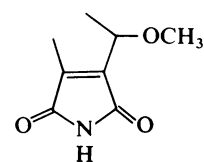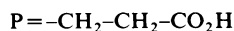
† Tentative linkage (see the text).

Scheme 1. *Proposed structures for protein-bound phycourobilin and its derivatives* Chromophore conjugated systems are emphasized by thickened lines.

dimethyl ester, yielded the imides methylpropyl-maleimide methyl ester and methylethylmaleimide in approximately equal proportions, demonstrating that this pigment was a mesobiliverdin. This verdin, as the dimethyl ester, was identified as mesobiliverdin IXα by the t.l.c. method of O'Carra & Colleran (1970). Since these conditions of release and conversion seem unlikely to cause any alteration in the carbon skeleton, these results indicate that phycourobilin is a tetrapyrrole, having a IXα skeletal structure containing no oxygenated bridge positions (Lemberg & Legge, 1949), and having an empirical formula identical with that of mesobiliverdin.

Arrangement of double bonds. The conjugated system, which is depicted by the heavy double lines, is in agreement with the urobilinoid spectral characteristics of the bilin (Table 1 and Fig. 2). The presence of the double bond (*) in ring A is consistent with the findings that phycourobilin could be converted, presumably by isomerization, into a red pigment (structure II, Scheme 1) having similar spectral properties to phycoerythrobilin in acid and alkali under O_2-free N_2 (see the Experimental section). The formation of this red pigment during acid $FeCl_3$ dehydrogenation could also be attributed to the isomerization of this double bond (*) into the bridge position under the strong acid conditions of the dehydrogenation reaction rather than to oxidation by the $FeCl_3$ reagent.

The presence of the double bond in ring D is supported by the observation that on chromic acid degradation this ring yields a maleimide rather than a succinimide (see below).

Chromic acid degradation studies. Chromic acid degradation of the phycourobilin-containing peptide at 20°C releases one imide, methylpropyl-maleimide (structure VI, Scheme 1). The second methylpropylmaleimide and the imide methylethyl-idinesuccinimide (structure V, Scheme 1) were released by subsequent hydrolysis at 100°C, indicating that phycourobilin, like phycoerythrobilin and phycocyanobilin (Rüdiger & O'Carra, 1969; Killilea et al., 1980), is linked to the polypeptide chain through the methylethylidinesuccinimide-yielding ring and through one of the inner, propionic acid-bearing, rings. Since phycourobilin was shown above to be a IXα-type bilin, the bound and unbound methylpropylmaleimide residues must derive from the two internal pyrrole rings, i.e. rings B and C.

Since phycourobilin is converted in acid or alkali (see the Experimental section) into phycoerythrobilin, or to a rhodenoid pigment with the same conjugated system, it seems that ring A of bound phycourobilin must be similar to that of bound phycoerythrobilin apart from the position of the shifting double bond involved in the interconver-
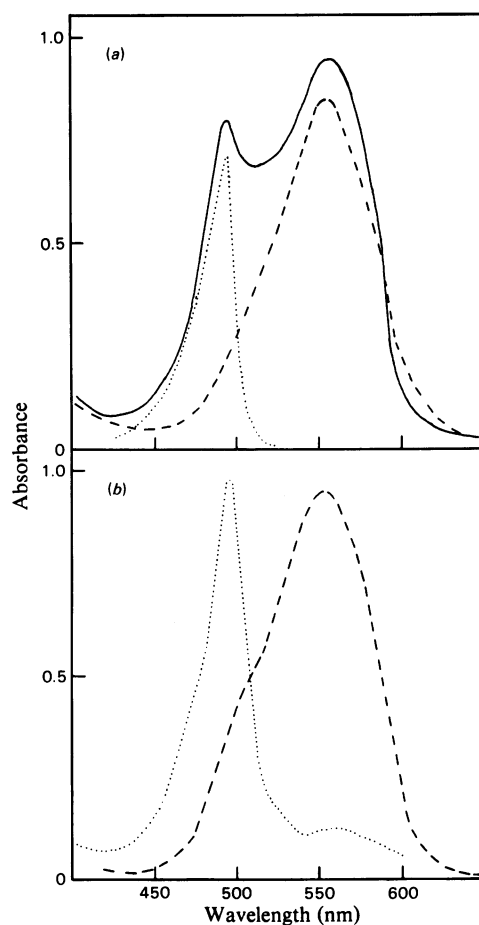

Fig. 2. *Absorption spectra in the visible region in 0.1 M-HCl of (a) denatured R-phycoerythrin (——), denatured C-phycoerythrin (— — —) and i-urobilin (······) and (b) phyco-erythrobilin-containing peptide (— — —) and phycourobilin-containing peptide (······)*

sion of the two pigments. Also, phycourobilin, like phycoerythrobilin (Rüdiger & O'Carra, 1969; Killilea et al., 1980), could be converted into a violinoid pigment (via a phycoerythrobilinoid/rhodenoid intermediate) in 1 M-KOH under N_2. This conversion would involve (a) the isomerization of the double bond (*) into the bridge position, (b) hydrolysis of the ring A-apoprotein linkage with the resultant formation of an unsaturated centre (structure II, Scheme 1) and (c) then the isomerization of this new double bond into the conjugated system to give the violinoid pigment (structure III, Scheme 1). In addition, phycourobilin, like phyco-erythrobilin and phycocyanobilin (Rüdiger & O'Carra, 1969), undergoes a spectral red-shift in alkali. This spectral characteristic has only been

observed with bilins having a pyrrole ring that yields methylethylidinesuccinimide on chromic acid degradation (Rüdiger & O'Carra, 1969). It is therefore probable that the methylethylidinesuccinimide produced from ring A of phycourobilin during chromic acid degradation arises by a mechanism similar to that postulated previously in relation to phycoerythrobilin (Killilea et al., 1980). The ring A linkage of phycourobilin shown in Scheme 1 is postulated on the basis of this reasoning.

These results accounted for three of the four pyrrole rings of phycourobilin. In view of the studies on the stability of the phycourobilin–apoprotein linkages, it was considered that the structure and attachment of the fourth ring, ring D, resembled that of the cysteine adduct of haem in cytochrome c, as postulated in structure I (Scheme I). Chromic acid degradation of this structure at 100°C would have been expected to cleave the thioether linkage at least partially and give rise to the imide, methyl-α-hydroxyethylmaleimide (structure VII, Scheme I). The release of pigment on refluxing the phycourobilin-containing peptide in 2M-HCl presumably entailed hydrolysis of the ring D linkage, but no methyl-α-hydroxyethylmaleimide was formed on chromic oxidation of this released pigment either. One possibility was that the imide was unstable in the chromic acid reagent and was further oxidized to unidentified non-imide product(s). Support for this possibility was the observation that methyl-α-hydroxyethylmaleimide was not found in the chromic acid degradation products of haematobiliverdin or its dimethyl ester, which contain two similar rings (only methylpropylmaleimide or its methyl ester could be detected).

On the other hand, the pigment released from the phycourobilin-containing peptide on methanolysis was found to yield a derivative of ring D on chromic acid degradation that was identified as methyl-α-methoxyethylmaleimide (structure VIII, Scheme 1), indicating methanolysis of the ring D linkage. The thioether bonds linking the biliverdin to the apoprotein in verdocytochrome c were also cleaved by methanolysis, and this released pigment also yielded methyl-α-methoxyethylmaleimide on chromic acid degradation.

On the basis of these comparative studies of the phycourobilin-containing peptide, cytochrome c and verdocytochrome c it is proposed that the third apoprotein linkage of phycourobilin involves a thioether linkage through the α-carbon atom of the C_2 side chain of ring D, as shown in Scheme 1. The release of the chromophore from the phycourobilin-containing peptide and its conversion into mesobiliverdin IXα (structure IV, Scheme 1) on refluxing in methanolic 1M-KOH under N_2 must

involve an elimination reaction at this C_2 thioether-linked side chain, resulting in the formation of a double bond, which then isomerizes into the ring system.

Amino acids involved in the phycourobilin–apoprotein linkages. In order to try to determine the amino acids involved in the linkages of phycourobilin to the peptide, phycourobilin-containing peptide material was digested with mammalian proteinases and the amino acid composition of the resulting chromopeptide material was determined as described previously for the other phycobilins (Killilea et al., 1980). The results are shown in Table 2. As reported previously for the other phycobilins, the incomplete hydrolysis by the peptidases to leave only the directly attached amino acid residues is attributed to steric hindrance by the bulky aromatic chromophore (Killilea et al., 1980). The chromopeptide material contained about 2 mol of cysteic acid/mol of chromophore. One of these cysteine residues is proposed to be linked through its thiol group to the α-carbon atom of the C_2 side chain of ring A, as previously proposed for phycoerythrobilin and phycocyanobilin (Crespi & Smith, 1970; Byfield & Zuber, 1972; Köst-Reyes et al., 1975; Williams & Glazer, 1978; Bryant et al., 1978; Muckle et al., 1978; Brown et al., 1979; Killilea et al., 1980; Lundell et al., 1984). The second cysteine residue would be involved in the proposed stable thioether linkage to the α-carbon atom of the C_2 side chain of ring D, as depicted in Scheme 1. Other evidence for the involvement of these cysteine residues in the linkage of the

Table 2. *Quantitative amino acid analysis of phycourobilin-containing 'minimal chromopeptides'*

Peptides were oxidized with performate before hydrolysis. Chromophore content is based on an ε of $55660M^{-1} \cdot cm^{-1}$ at 495nm for phycourobilin. Values of serine and threonine were corrected for destruction during hydrolysis, assuming 10% and 5% loss respectively (Moore & Stein, 1963).

Amino acid	Content (mol/mol of chromophore)
Cysteic acid	1.99
Glutamic acid	0.47
Aspartic acid	0.63
Serine	0.45
Threonine	0.24
Glycine	1.37
Alanine	0.42
Proline	0.98
Valine	0.95
Leucine	0.12
Isoleucine	0.08
Lysine	0.55

phycourobilin to the peptide was the apparent inaccessibility of these thiol groups to S-amino-ethylation. The R-phycoerythrin preparations were subjected to prior S-aminoethylation before digestion with trypsin, but the cysteine residues in the chromopeptide material were not modified and were released as cysteic acid after performate oxidation and hydrolysis. Such procedures do not convert S-aminoethylcysteine into cysteic acid (S. D. Killilea & P. J. MacGillivray, unpublished work).

Lundell et al. (1984) have reported that one of the three phycoerythrobilin residues in the β-subunit of B-phycoerythrin is linked to the polypeptide chain through rings A and D to two cysteine residues, as reported here for phycourobi-lin. However, no precautions (Killilea et al., 1980) were taken in the preparation of the phycoerythro-bilin-peptide material to prevent possible artifact covalent linkage between the vinyl side chain group of ring D of phycoerythrobilin and a free thiol group on the polypeptide (O'Carra et al., 1964; Rüdiger & O'Carra, 1969).

The chromic acid degradation studies indicated that phycourobilin, like phycoerythrobilin and phycocyanobilin, is also linked to the polypeptide chain through one of the inner propionic acid-bearing rings. It was not possible to determine through which of these inner rings the chromo-phore is linked to the peptide, and the linkage is shown through ring C (structure I, Scheme 1). As in the case of phycoerythrobilin and phycocyano-bilin (Killilea et al., 1980), the linkage through ring C is postulated to involve the hydroxy side-chain group of serine in an ester linkage with the carboxylic group of the propionic side chain of phycourobilin. The possibility of the ε-amino side-chain group of lysine being involved in a peptide linkage with the carboxy group has been previously ruled out (O'Carra et al., 1964). This linkage cannot involve the ring nitrogen, as phycourobilin in situ forms a zinc complex (O'hEocha & O'Carra, 1961; Table 1). The α-subunit and β-subunit of R-phycoerythrin contain one phycourobilin residue each (S. D. Killilea, D. N. Nolan & P. O'Carra, unpublished work), but only about 0.5 mol of serine was determined per mol of phycourobilin in the phycourobilin 'minimal peptide' material (Table 2). Thus either only one of the two phycourobilin residues is linked to a serine residue or the low yield of serine was due to cleavage of some of the ester linkages during preparation of the 'minimal chromopeptide' material. Other studies, however, have raised doubts about this ester linkage between the hydroxy group of a serine residue and the carboxy side chain of one of the inner propionic acid-bearing rings of the phycobilins (Crespi &

Smith, 1970; Byfield & Zuber, 1972; Williams & Glazer, 1978; Bryant et al., 1978; Troxler et al., 1978; Brown et al., 1979). Sequence studies of the multiple phycocyanobilin-containing sites in C-phycocyanin and the phycoerythrobilin-contain-ing sites in C- and B-phycoerythrins have revealed that a serine residue is found close to the cysteine residue involved in the ring A linkage in less than half of the sites in each of these biliproteins (Bryant et al., 1978; Frank et al., 1978; Muckle et al., 1978; Williams & Glazer, 1978; Lundell et al., 1984). The possibility could not be ruled out, however, that this serine or serine residues at other sites in the polypeptide chains were linked to the chromo-phores and that these linkages were cleaved in the preparation and/or sequencing of the chromo-peptide fractions.

The argument favouring linkage through one of the inner rings was based on chromic acid degradation studies on the protein-bound chromo-phores (Rüdiger & O'Carra, 1969; Köst et al., 1975; the present work). Troxler et al. (1978), on the other hand, could not repeat the results obtained by Rüdiger & O'Carra (1969). Although the reason for this discrepancy is not entirely clear, the different results obtained by Troxler et al. (1978) may have been due to the shorter reaction times and the more dilute reagents used. Indeed these workers used phycocyanobilin preparations radiolabelled in vivo and did not observe quantita-tive release of the imides under the conditions of their chromic acid degradation procedure, but a time course of the conversion of the phycobilin into imide products was not reported. Other evidence favouring the linkage to one of the inner rings has been obtained from studies on the stability of the phycobilin–apoprotein linkages in R-phyco-erythrin in alkali. In alkali the chromophores of R-phycoerythrin are converted into violinoid pig-ment (Rüdiger & O'Carra, 1969; Killilea et al., 1980; the present work). Detailed studies of this conversion has revealed that cleavage of the ring A–apoprotein linkage must take place prior to conversion of the chromophores into the violin (Rüdiger & O'Carra, 1969; Killilea et al., 1980). Much of this violin remains attached to the polypeptide, and part of this pigment can be released by hydrolysis with HCl at room tempera-ture (Rüdiger & O'Carra, 1969). The acid-released violin derives from the phycoerythrobilin residues, and the acid-resistant violin derives from the phycourobilin chromophores, which as shown above are not released by this acid treatment. Thus, until these discrepancies between the sequence studies and those just described are explained, the possibility that some or all of the phycobilins in individual biliproteins might be linked to the protein via an ester linkage though

one of the inner rings cannot be ruled out. For this reason we have tentatively included this linkage in structure I (Scheme 1).

These results and a previous report (Killilea et al., 1980) detail the elucidation of the structures and apoprotein linkages of the three bilin chromophores found in the photosynthetically active biliproteins in red and blue–green algae.

References

Brown, A. S., Offner, G. D., Ehrhardt, M. M. & Troxler, R. F. (1979) *J. Biol. Chem.* **254**, 7803–7811

Bryant, D. A., Hixson, C. S. & Glazer, A. N. (1978) *J. Biol. Chem.* **253**, 220–225

Byfield, P. G. H. & Zuber, H. (1972) *FEBS Lett.* **28**, 36–40

Chapman, D. J., Cole, W. J. & Siegelman, H. W. (1968) *Phytochemistry* **7**, 1831–1835

Crespi, H. L. & Smith, U. H. (1970) *Phytochemistry* **9**, 205–212

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, Elsevier Publishing Co., Amsterdam

Ficken, G. E., Johns, R. B. & Linstead, R. P. (1956) *J. Chem. Soc.* 2272–2280

Frank, G., Sidlee, W., Widmer, H. & Zuber, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1491–1507

Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 386–390

Killilea, S. D. & O'Carra, P. (1971) *J. Chromatogr.* **54**, 284–285

Killilea, S. D. & O'Carra, P. (1978) *J. Chromatogr.* **166**, 338–340

Killilea, S. D., O'Carra, P. & Murphy, R. F. (1980) *Biochem. J.* **187**, 311–320

Köst, H.-P., Rudiger, W. & Chapman, D. J. (1975) *Justus Liebigs Ann. Chem.* 1582–1593

Köst-Reyes, E., Köst, H.-P. & Rüdiger, W. (1975) *Justus Liebigs Ann. Chem.* 1594–1600

Lemberg, R. & Legge, J. W. (1949) *Haematin Compounds and Bile Pigments*, Interscience, New York

Levin, E. Y. (1966) *Biochemistry* **5**, 2845–2852

Liu, T. Y., Stein, W. H., Moore, S. & Elliott, S. D. (1965) *J. Biol. Chem.* **240**, 1143–1149

Lundell, D. J., Glazer, A. N., DeLange, R. J. & Brown, D. M. (1984) *J. Biol. Chem.* **259**, 5472–5480

Moore, S. & Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831

Muckle, G., Otto, J. & Rüdiger, W. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 345–355

O'Carra, P. (1965) *Biochem. J.* **94**, 171–174

O'Carra, P. & Colleran, E. (1970) *J. Chromatogr.* **50**, 458–468

O'Carra, P. & O'hEocha, C. (1966) *Phytochemistry* **5**, 993–997

O'Carra, P., O'hEocha, C. & Carroll, D. M. (1964) *Biochemistry* **3**, 1343–1350

O'Carra, P., Murphy, R. F. & Killilea, S. D. (1980) *Biochem. J.* **187**, 303–309

O'hEocha, C. & O'Carra, P. (1961) *J. Am. Chem. Soc.* **83**, 1091–1093

Paul, K. G. (1950) *Acta Chem. Scand.* **4**, 239–245

Raftery, M. A. & Cole, R. D. (1966) *J. Biol. Chem.* **241**, 3457–3461

Reindel, F. & Hoppe, W. (1954) *Chem. Ber.* **87**, 1103–1108

Rüdiger, W. & O'Carra, P. (1969) *Eur. J. Biochem.* **7**, 509–516

Troxler, R. F., Brown, A. S. & Köst, H.-P. (1978) *Eur. J. Biochem.* **87**, 181–189

Watson, C. J., Weimer, M., Petryka, Z. J., Lightner, D. A., Moscowitz, A., Davis, E. & Beach, N. A. (1969) *Arch. Biochem. Biophys.* **131**, 414–422

Williams, V. P. & Glazer, A. N. (1978) *J. Biol. Chem.* **253**, 202–211